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Rate and characterization of parasitic infestation in a stool specimens in children with diarrhea by using polymerase chain reaction technique

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Abstract

Diarrheal disorders are a major health problem in pediatrics worldwide. Accounting for more than 750,000 deaths in children under the age of 5 per year, they are the second leading cause of death in this population according to the World Health Organization (WHO). *Giardia duodenalis* (*G. duodenalis*), *Cryptosporidium parvum* (*C. parvum*) and *Entamoeba histolytica* (*E. histolytica*) are the most common protozoan parasites that cause acute diarrhoeal illnesses in children. For many decades, the laboratory diagnosis of intestinal amebiasis has been based upon the microscopic examination of stool samples. So the aim of the present study was to compare the sensitivity of light microscopic method with the PCR method to identify parasitic pathogens among children with persistent diarrhea. 90 stool samples were collected from children that infected by diarrhea from Wasit hospitals and were analyzed by routine light microscopic method and PCR techniques. In conclusion, parasitic cause of persistent diarrhea is underestimated when diagnosed solely by microscopic techniques and that mixed infection are going to be missed and the use of PCR is helpful in avoiding these problems**.**

Key words: *Giardia lamblia*, *Cryptosporidium parvum*, *Entamoeba histolytica*, PCR, diarrhea

Introduction

Diarrheal disorders are a major health problem in pediatrics worldwide. Accounting for more than 750,000 deaths in children under the age of 5 per year, they are the second leading cause of death in this population according to the World Health Organization (WHO) [(1)]. According to the volume, diarrhea is defined as loose stools of >10 mL/kg/day in infants and >200 g/24 hr in older children [(2)]. It is difficult to accurately measure the volume of the stool in clinical environment. Therefore, diarrhea may be defined as the passage of ≥3 watery stools per day in the United Nations International Children's Emergency Fund and the World Health Organization (WHO) [(3)]. Persistent diarrhea is an episode of diarrhea of infectious etiology, which develops acutely but continues for more than 14 days. Chronic diarrhea is defined as diarrhea that lasts for more than 14 day and is usually not associated with only infectious cause, and associated with various causes and malabsorption [(4)]. An accurate diagnosis and the proper treatments are very important because chronic diarrhea effects physical and psychological development in children. The causes of chronic diarrhea are divided into infectious and noninfectious etiologies. The most frequent cause of chronic diarrhea is enteric infection in both in developing and industrialized countries [(5)]. Intestinal parasitic infections (IPIs) are the most common infections among children in developing countries. *Giardia duodenalis* (*G. duodenalis*), *Cryptosporidium parvum* (*C. parvum*) and *Entamoeba histolytica* (*E. histolytica*) are the most common protozoan parasites that cause acute diarrhoeal illnesses in children [(6)]. Until 1993, the studies on the prevalence of amoebiasis showed that approximately 10% of the world's population was infected with *Entamoeba histolytica*. However, only 1% of these infections developed into an invasive form of the disease, with a mortality factor of about 100,000 cases per year [(7)]

Entamoeba histolytica, *Giardia lamblia* (synonymous with *G. intestinalis* and *G. duodenalis*), and *Cryptosporidium* spp. are the causative agents of amebiasis, giardiasis, and cryptosporidiosis, respectively. These organisms are among the most common intestinal protozoa associated with diarrhea in developed and developing countries [(8, 9)]. The Global Burden of Disease Study (GBDS) estimated that amebiasis was associated with more than 55,000 deaths and 2.2 million disability-adjusted life years (DALYs), and cryptosporidiosis with more than 99,000 deaths and 8.3 million DALYs in 2010 [(10, 11)]. Most of these deaths and DALYs occurred in developing countries. Adequate knowledge of the geographical distribution of parasites and the demographic variables that influence their prevalence is important for effective control of infection in at-risk populations [(12)]. For many decades, the laboratory diagnosis of intestinal amebiasis has been based upon the microscopic examination of stool samples and hence widely known as the 10% disease. However, the recent description of various

nonpathogenic *Entamoeba* species like *Entamoeba dispar*, *E. moshkovskii* and the newly described *Entamoeba bangladeshi* as morphologically indistinguishable forms from *E. histolytica* has required the need for alternative diagnostic methods for differentiation [13]. *G. lamblia* have seven complex genotypes termed (A-H). Genotypes A and B are the main and only causes of human infections and PCR technique has been shown to have better sensitity in detecting *G. lamblia* infection than routine microscopic methods [14]. Common methods for detection of *Cryptosporidium* are parasite visualization using acid-fast staining as well as fluorescent staining after concentration. Because they are obligate intracellular parasites, cultivating of the organism is not routine in the laboratory. To obtain high sensitivity in the diagnosis with microscope, a modified Ziehl- Neelsen staining and a minimum amount of 500,000 oocysts in each gram of examined stool required. Besides, identification of oocysts in direct microscopic detection is depended to the time as well as experience of stool examiner. The PCR techniques have proved both specific and sensitive methods for detection of protozoan infections and *Cryptosporidium* specimen types [15]. So the aim of the present study was to compare the sensitivity of light microscopic method with the PCR method to identify parasitic pathogens among children with persistent diarrhea.

Patients, materials and methods

Feces sample collection.

90 stool samples were collected from children that infected by diarrhea from Wasit hospitals. The Fecal sample was transferred to a clean, dry plastic container and transported to the laboratory for analysis.

Genomic DNA Extraction

Genomic DNA was extracted from feces samples by using (Stool DNA extraction Kit, Bioneer. Korea). The extraction was done according to company instructions by using stool lysis protocol method with Proteinase K. After that, the extracted gDNA was checked by Nanodrop spectrophotometer, and then stored at -20C at refrigerator until used in PCR amplification.

Polymerase chain reaction

PCR assay was performed for direct detection of Giardia intestinalis, Entamoeba histolytica , and Cryptosporidium parvum by using specific primer for small subunit ribosomal RNA gene, the primers were designed in this study using NCBI-Genbank and Primer3plus and provided by (Bioneer company . Korea) as following table 1.

Table 1: DNA primers

Primer	Sequence (5'-3')		Amplicon	Genbank
G.intestinalis	F	CTGGCCCAAGAGTCCTCAAG	488bp	U09491.1
	R	CCGGAGTCGAACCCTGATTC		
E.histolytica	F	ATTGGAGGGCAAGTCTGGTG	389bp	GQ423748.1
	R	AAATGCTTTCGCTCTCGTGC		
C. parvum	F	ATTGGAGGGCAAGTCTGGTG	242bp	HQ259571.1
	R	CCATGCTGGAGTATTCAAGGC		

Then PCR master mix was prepared by using (AccuPower® PCR PreMix kit. Bioneer. Korea). The PCR premix tube contains freeze-dried pellet of (Taq DNA polymerase 1U, dNTPs 250µM, Tris-HCl (pH 9.0) 10mM, KCl 30mM, MgCl2 1.5mM, stabilizer, and tracking dye) and the PCR master mix reaction was prepared according to kit instructions in 20µl total volume by added 5µl of purified genomic DNA and 1.5µl of 10pmole of forward primer and 1.5µl of 10pmole of reverse primer, then complete the PCR premix tube by deionizer PCR water into 20µl and briefly mixed by Exispin vortex centrifuge (Bioneer. Korea). The reaction was performed in a thermocycler (Mygene, Bioneer. Korea) by set up the following thermocycler conditions; initial denaturation temperature of 95 °C for 5 minutes followed by 30 cycles at denaturation 95°C for 30 seconds, annealing 58°C for 30 seconds, and extension 72°C for 1 min. minute and then final extension at 72°C for 5 minutes. The PCR products were examined by electrophoresis in a 2% agarose gel, stained with ethidium bromide, and visualized under UV illumination.

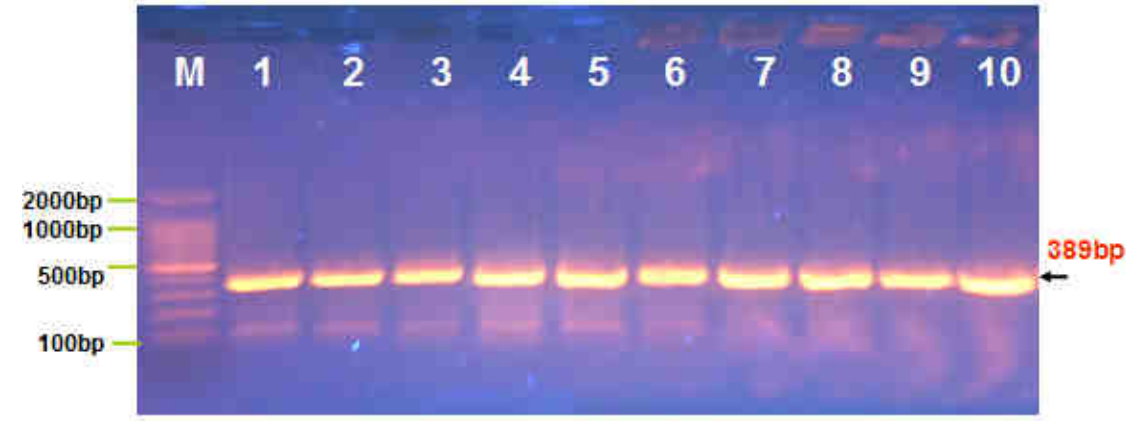

Figure 1: Agarose gel electrophoresis image that show the PCR product of small subunit ribosomal RNA gene that using in detection Entamoeba histolytica in human stool samples. Where M: Marker (2000-100bp), lane (1-10) some positive samples at 389bp PCR product size.

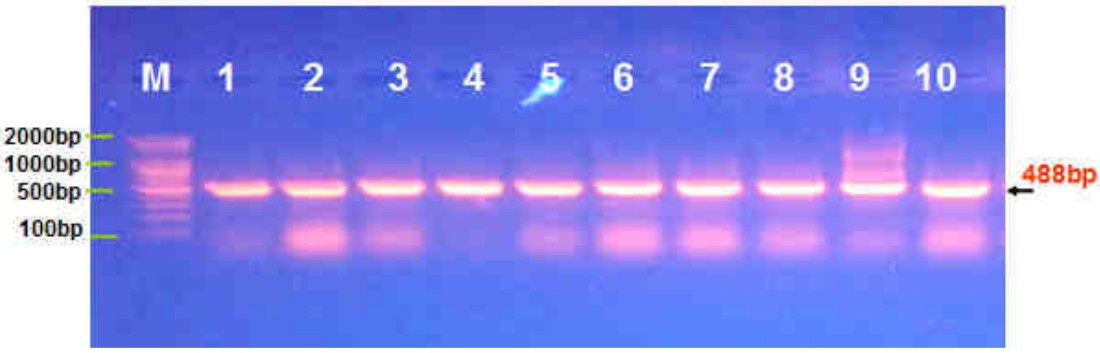

Figure 2: Agarose gel electrophoresis image that show the PCR product of small subunit ribosomal RNA gene that using in detection Giardia intestinalis in human stool samples. Where M: Marker (2000-100bp), lane (1-10) some positive samples at 488bp PCR product size.

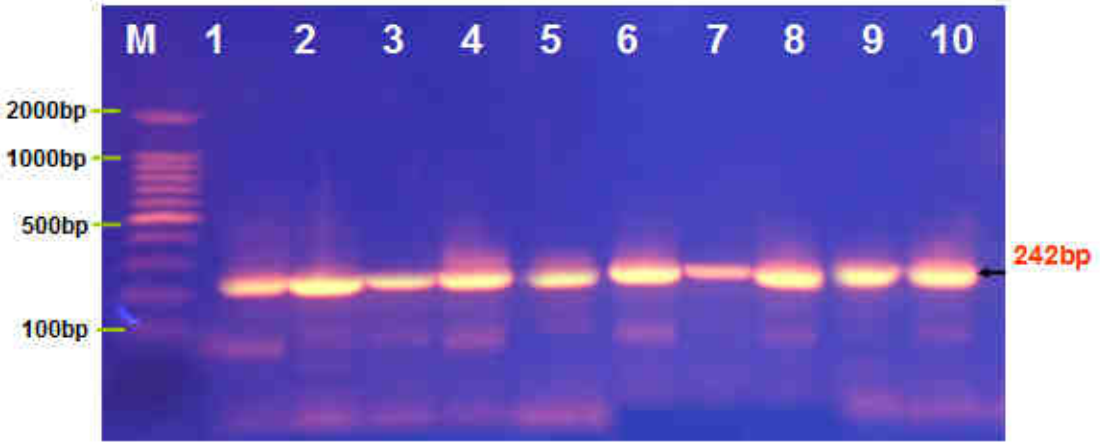

Figure 3: Agarose gel electrophoresis image that show the PCR product of small subunit ribosomal RNA gene that using in detection Cryptosporidium parvum in human stool samples. Where M: Marker (2000-100bp), lane (1-10) some positive samples at 242bp PCR product size.

Results

Table 2: Rate of parasite detection by light microscope and PCR methods

Cases with parasite detection	PCR	Light microscope	P*
Positive n (%)	90 (81.1)	54 (48.6)	<0.001 HS
Negative n (%)	21 (18.9)	57 (51.4)	
Total cases n (%)	111 (100.0)	111 (100.0)	

*McNemar test

Table 3: Mixed versus pure infection

Cases with parasite detection	PCR	Light microscope
Pure n (%)	89 (80.2)	54 (48.6)
Mixed n (%)	11 (9.9)	0 (0.0)
Negative n (%)	21 (18.9)	57 (51.4)
Total n (%)	111 (100.0)	111 (100.0)

Table 4: Number and percentage of individual parasites according to method of detection

Parasite	PCR	Light Microscope
E. histolytica n (a, b, c)	64 (71.1 %, 57.7 %, 52.5 %)	40 (74.1 %, 36.0 %, 74.1 %)
G. lamblia n (a, b, c)	39 (43.3 %, 35.1 %, 32.0 %)	14 (25.9 %, 12.6 %, 25.9 %)
Cryptospordium parvum n (a, b, c)	19 (21.1 %, 17.1 %, 15.5 %)	0 (0.0 %, 0.0 %, 0.0 %)
Total cases with parasites *n*	90	54
Total cases with diarrhea *n*	111	111
Total isolated parasites *n*	122	54

n: number; a: % out of Total cases with parasites; b: % out of Total cases with diarrhea; c: % out of Total isolated parasites

Discussion

Diarrhea is common cause for hospital admission in Iraq and usually associated with variable degree of dehydration that require prompt management through fluid and electrolyte replacement [16]. Parasites and especiilay E. hystolytica and G. lamblia are among common pathogens that are detected routinely in children presented with diarrheal disease in Iraq; however the usual mode of detection is by routine light microscopic technique [16, 17]. This technique requires the existence of the parasite in sufficient concentration in examined stool for proper detection, in addition, the technique require good experience possessed by the examining technician [15]. Application of DNA based PCR techniques in diagnosing infectious agents replaced the routine light microscopic methods in substantial number of developed countries. PCR method has been proven to have superior sensitivity and specificity in detecting microbial agents including parasites in comparison with routine light microscopic methods [15]. Our results has shown great discrepancy in the percentage of E. histolytica, G.lamblia and C. parvum detected in stool samples of children enrolled in the present study and that PCR was

proved to be more sensitive in this regard. It was also surprising that PCR detected cases with mixed parasitic infestation whereas the routine light microscopic techniques were unable to detect mixed infections. This may be due to the satisfaction of the technician with one detected pathogen so that more meticulous effort was not applied to identify the possibility of the presence of other pathogen in the same specimen.

In accordance with present study, a lot of published literature documented the better sensitivity of DNA based technologies in comparison with light microscopic techniques when dealing with infectious diarrhea [18, 19].

Another important point to be discussed is that those parasites have different genetically distinct strains and not all of them are pathogenic, and these strains cannot be identified by light microscopic techniques because of the great morphologic similarities [13]. So one should be careful when labeling a patient to have a parasitic cause of diarrhea, while using microscopic techniques only. In conclusion, parasitic cause of persistent diarrhea is underestimated when diagnosed solely by microscopic techniques and that mixed infection are going to be missed and the use of PCR is helpful in avoiding these problems.

Conclusion: parasitic cause of persistent diarrhea is underestimated when diagnosed solely by microscopic techniques and that mixed infection are going to be missed and the use of PCR is helpful in avoiding these problems**.**

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